



# Chinese Patent Medicine Liuweiwuling Tablet had Potent Inhibitory Effects on Both Wild-Type and Entecavir-Resistant Hepatitis B Virus (HBV) *in vitro* and Effectively Suppressed HBV Replication in Mouse Model

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### \*Correspondence:

Zhao-fang Bai  
baizf2008@hotmail.com  
Yan Liu  
liuyan5360@163.com  
Xiao-he Xiao  
pharmacy302xxh@126.com

†These authors have contributed  
equally to this work

### Specialty section:

This article was submitted to  
Pharmacology of Infectious Diseases,  
a section of the journal  
Frontiers in Pharmacology

Received: 11 August 2021

Accepted: 06 October 2021

Published: 27 October 2021

### Citation:

Ge F-I, Si L-I, Yang Y, Li Y-h, Lv Z-I,  
Liu W-h, Liao H, Wang J, Zou J, Li L,  
Li H, Zhang Z-I, Wang J-b, Lu X-c,  
Xu D-p, Bai Z-f, Liu Y and Xiao X-h  
(2021) Chinese Patent Medicine  
Liuweiwuling Tablet had Potent  
Inhibitory Effects on Both Wild-Type  
and Entecavir-Resistant Hepatitis B  
Virus (HBV) *in vitro* and Effectively  
Suppressed HBV Replication in  
Mouse Model.  
Front. Pharmacol. 12:756975.  
doi: 10.3389/fphar.2021.756975

Fei-lin Ge<sup>1,2†</sup>, Lan-lan Si<sup>2†</sup>, Yan Yang<sup>3†</sup>, Yuan-hua Li<sup>3†</sup>, Zhong-lin Lv<sup>4</sup>, Wen-hui Liu<sup>5</sup>,  
Hao Liao<sup>2</sup>, Jun Wang<sup>2</sup>, Jun Zou<sup>2</sup>, Le Li<sup>2</sup>, Hui Li<sup>3</sup>, Zi-lin Zhang<sup>2</sup>, Jia-bo Wang<sup>3</sup>, Xue-chun Lu<sup>4</sup>,  
Dong-ping Xu<sup>2</sup>, Zhao-fang Bai<sup>3\*</sup>, Yan Liu<sup>2\*</sup> and Xiao-he Xiao<sup>3\*</sup>

<sup>1</sup>School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China, <sup>2</sup>Department of Infectious Diseases, The Fifth Medical Center of Chinese PLA General Hospital, Beijing, China, <sup>3</sup>Department of Liver Diseases, The Fifth Medical Center of Chinese PLA General Hospital, Beijing, China, <sup>4</sup>Department of Hematology, The Second Medical Center and National Clinical Research Center for Geriatric Diseases, Chinese PLA General Hospital, Beijing, China, <sup>5</sup>Department of Gastroenterology, The Second Medical Center and National Clinical Research Center for Geriatric Diseases, Chinese PLA General Hospital, Beijing, China

Liuweiwuling Tablet (LWWL) is a licensed Chinese patent medicine (approval number: Z20060238) included in the national health insurance for anti-inflammation of chronic HBV infection, whereas its anti-HBV effect remains clarification. The study aimed to clarify its antiviral effect and related mechanisms. HepG2.2.15 cells (wild-type HBV-replicating cells) and HepG2. A64 cells (entecavir-resistant HBV-replicating cells) were used for *in vitro* test. Hydrodynamic injection-mediated HBV-replicating mouse model was used for *in vivo* test. Active compounds and related mechanisms for antiviral effect of LWWL were analyzed using network pharmacology and transcriptomics. The inhibition rates of LWWL (0.8 mg/ml) on HBV DNA, HBsAg, and pgRNA were 57.06, 38.55, and 62.49% in HepG2.2.15 cells, and 51.57, 17.57, and 53.88% in HepG2. A64 cells, respectively. LWWL (2 g kg<sup>-1</sup> d<sup>-1</sup> for 4 weeks)-treated mice had 1.16 log<sub>10</sub> IU/mL decrease of serum HBV DNA, and more than 50% decrease of serum HBsAg/HBeAg and hepatic HBsAg/HBcAg. Compared to tenofovir control, LWWL was less effective in suppressing HBV DNA but more effective in suppressing HBV antigens. Thirteen differentially-expressed genes were found in relation to HBV-host interaction and some of them were enriched in interferon (IFN)-β pathway in LWWL-treated HepG2.2.15 cells. CD3<sup>+</sup>CD4<sup>+</sup> T-cell frequency and serum IFN-γ were significantly increased in LWWL-treated mice compared to LWWL-untreated mice. Among 26 compounds with potential anti-HBV effects that were predicted by network pharmacology, four compounds (quercetin, luteolin, wogonin, and kaempferol) were experimentally confirmed to have antiviral potency. In conclusion, LWWL had potent

inhibitory effect on both wild-type and entecavir-resistant HBV, which might be associated with increasing IFN- $\beta$  and IFN- $\gamma$  production.

**Keywords:** hepatitis B virus, entecavir resistance, Chinese patent medicine, antiviral activity, active compounds

## INTRODUCTION

Hepatitis B virus (HBV) infection can cause chronic hepatitis B (CHB), increase occurrence risk of liver cirrhosis and hepatocellular carcinoma (HCC). An estimated 257 million individuals live with HBsAg positive, leading to more than 887,000 deaths annually (Revill et al., 2019). Two classes of anti-HBV agents, i.e., interferon (IFN) and nucleoside/nucleotide analogues (NAs), have been approved for treatment of HBV-infected diseases. So far, six NAs are licensed in China for the treatment of HBV-related diseases, including lamivudine (LAM), adefovir dipivoxil (ADV), telbivudine (LdT), entecavir (ETV), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). These antiviral agents have brought great benefit for patients, while a major challenge is that HBV is hardly to be eliminated from patients with chronic HBV infection using current anti-HBV agents. In addition, HBV drug-resistance and adverse drug reactions (ADRs) are also factors influencing therapeutic efficacy. Therefore, it is still urgent to develop novel efficacious drugs and therapies to improve clinical cure of chronic hepatitis B (Wang et al., 2019; Seo et al., 2020; Helen et al., 2013; Kim et al., 2017).

A few of traditional medicine (TM) and related components were documented with anti-HBV effects, such as *schisandrae chinensis fructus*, *salviae miltiorrhizae radix et rhizom*, *sophorae flavescens radix*, *tsaoko fructus*, wogonin, baicalein, and matrine (Liu et al., 2018; Si et al., 2019; Hepatobiliary Specialized Committee of China Association of Chinese Medicine and Liver Diseases Specialized Committee of China Medical Association of Minorities., 2018). Liuweiwuling Tablet (LWWL) is a drug approved by the China National Medical Products Administration (approval number: Z20060238) and it has been taken into the national health insurance. LWWL consists of six herbs, i.e., *schisandrae chinensis fructus* (*Wu Wei Zi* or WWZ), *ligustri lucidi fructus* (*Nv Zhen Zi* or NZZ), *forsythiae fructus* (*Lian Qiao* or LQ), *curcumae rhizoma* (*E Zhu* or EZ), *field sowthistle herb* (*Qu Mai Cai* or QMC), and *ganoderma spore* (*Ling Zhi Bao Zi Fen* or LZ). LWWL has good efficacy on anti-inflammation of chronic HBV infection (Hepatobiliary Specialized Committee of China Association of Chinese Medicine and Liver Diseases Specialized Committee of China Medical Association of Minorities., 2018; Hepatobiliary Specialized Committee of China Association of Chinese Medicine et al., 2020). In addition, a few of clinical reports indicated that LWWL combined with NAs could accelerate HBV DNA undetectability and normalization of alanine aminotransferase (ALT) compared to single NAs use (Zhao et al., 2016; Shanguan et al., 2016; Wang, 2014; Wang, 2020). However, the anti-HBV effects of LWWL and related mechanisms remains clarification. In light of the revelation, this study aimed to clarify anti-HBV effect and related mechanisms, and to identify major active compounds.

## MATERIALS AND METHODS

### Cell Lines and Cytotoxicity Assay

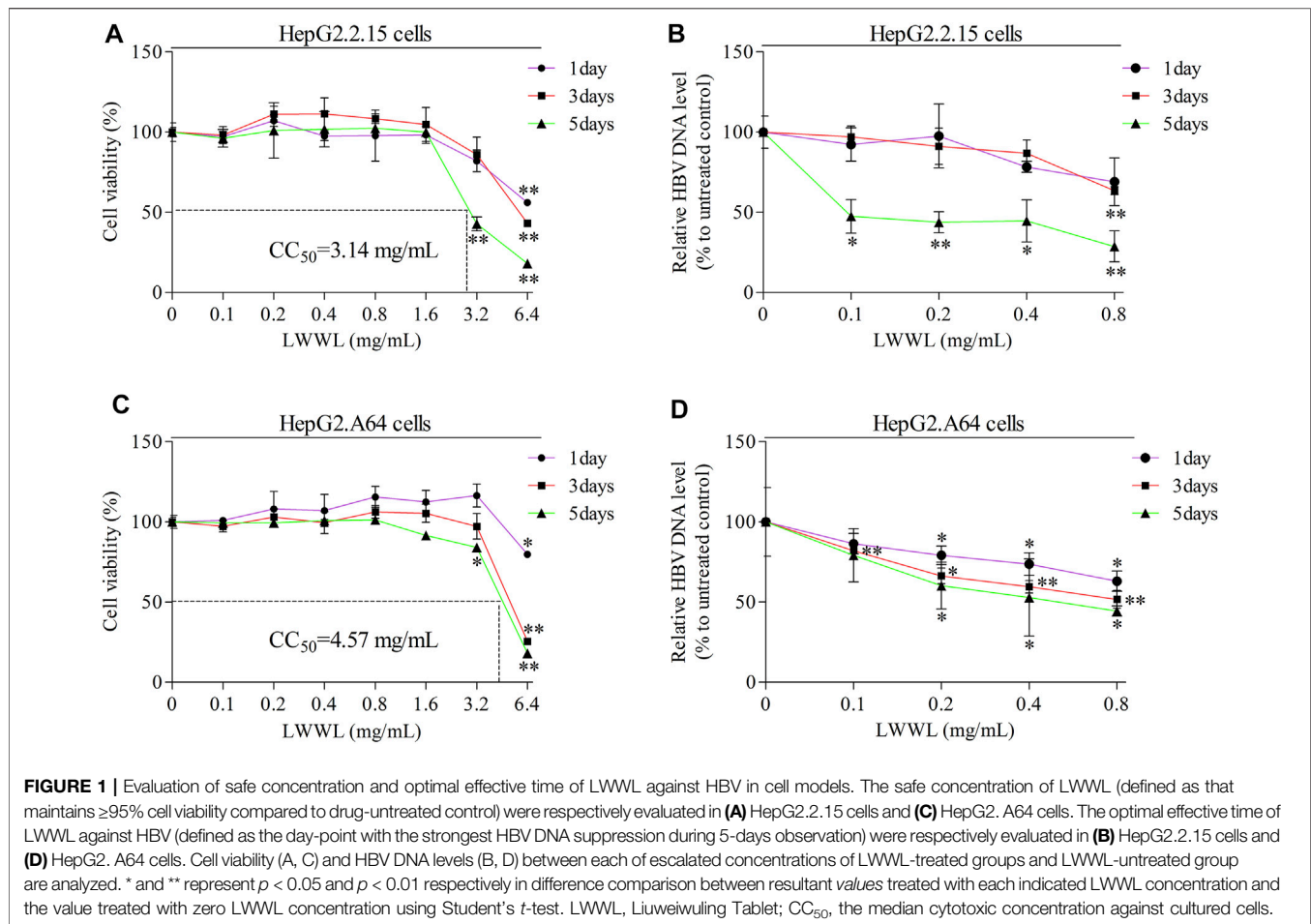
Two HBV-replicating cell lines HepG2.2.15 and HepG2.A64 were employed in the study. HepG2.A64 as an ETV-resistant HBV-replicating cell line has been employed previously (Liu et al., 2016; Liu et al., 2018). Compared to HepG2.2.15 cells, HepG2.A64 cells generated comparable HBV DNA, higher HBsAg but lower HBeAg. The cytotoxicity of LWWL (Shibo Jindu, Zibo, China) and four compounds quercetin, luteolin, wogonin, and kaempferol (purchased from MedChemExpress Co., Ltd., Monmouth Junction, United States) on cells were analyzed using Cell Counting Kit-8 (Dojindo Laboratories, Kyushu, Japan) according to the manufacturer's instructions. The median cytotoxic concentration (CC<sub>50</sub>) was calculated. The molecular and cellular studies were carried out in Biosafety level-2 (BSL-2) laboratory at Center Laboratory, The Fifth Medical Center of Chinese PLA General Hospital. All manipulations were strictly conducted according to the instructions of the laboratories.

### Evaluating Anti-HBV Activity of LWWL in Cell Models

HepG2.2.15 cells and HepG2.A64 cells were respectively plated into 48-well culture plates ( $2 \times 10^4$  cells/well). The cells in duplicate wells were treated with different concentrations of the drug (0, 0.1, 0.2, 0.4 and 0.8 mg/ml of LWWL, or 0, 0.2, 2, 20, 200  $\mu$ mol/L of TDF, or selective concentrations of compounds identified from LWWL) for 5 days. Culture supernatants were harvested in 5 days for determining HBsAg and HBeAg levels by ELISA kits (Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China), HBV pregenomic RNA (pgRNA) and HBV DNA levels were respectively determined by quantitative reverse-transcription PCR (qRT-PCR) and quantitative PCR (qPCR) assays as previously described (Ji et al., 2011; Liu et al., 2015; Wang et al., 2017). Half maximal inhibitory concentration (IC<sub>50</sub>) were calculated. The experiments were performed for three times independently.

### Establishment of HBV Mouse Model With Hydrodynamic Injection of Adeno-Associated Virus Plasmid (pAAV)-HBV1.2

The pAAV carrying 1.2-mer wild-type HBV genome was obtained from P.J. Chen (National Taiwan University, Taipei). C57BL/6 male mice weighted 20–22 g were injected into tail vein with 20  $\mu$ g of pAAV-HBV1.2 plasmid in 2 ml PBS within 6–8 s. In 3 days, the mice were bled through orbit for monitoring HBsAg, HBeAg and HBV DNA levels.



## Evaluating Anti-HBV Activity of LWWL in the Mouse Model

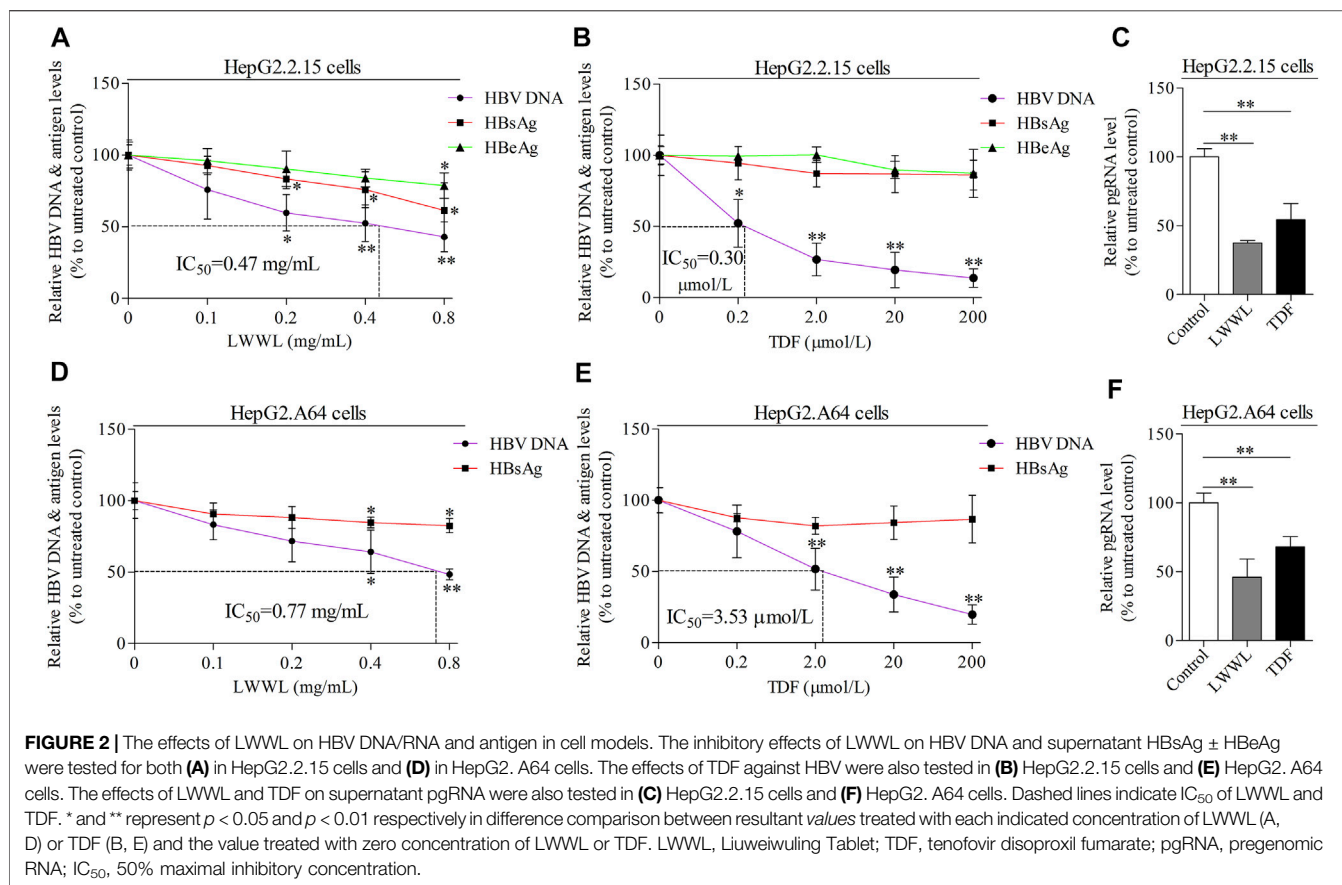
The pAAV-HBV1.2-replicating mice were divided into four groups with six mice each group as follows: normal saline (NS) group, low-dose LWWL ( $1 \text{ g kg}^{-1} \text{ d}^{-1}$ ) group, high-dose LWWL ( $2 \text{ g kg}^{-1} \text{ d}^{-1}$ ) group, and TDF ( $63 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) group. Intraperitoneal injection was conducted once a day for 4 weeks. The mice were bled weekly during treatment through orbit. The mice serum were harvested to measure HBV DNA, HBsAg, HBeAg, and IFN- $\gamma$  using ELISA kits (Multi Sciences Co., Ltd., Hangzhou, China) according to the manufacturer's instructions. Hepatic HBeAg and HBsAg were examined using immunohistochemical staining of paraffin-embedded tissue. Monoclonal mouse anti-HBs (MXB Biotechnologies, Fuzhou, China) and monoclonal mouse anti-HBe (Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China) were used for the examination. The animal study was conducted in BSL-2 laboratory at Animal Experimental Center, The Fifth Medical Center of Chinese PLA General Hospital. All manipulations were strictly conducted according to the instructions of the laboratories. The study protocol was approved by the Committee on the Ethics of Animal Experiments of The Fifth Medical Center of Chinese PLA General Hospital (Permit number: IACUC-2021-0009).

## Flow Cytometric Analysis

Effects of LWWL on splenic T cells activities were investigated for the high-dose LWWL mice ( $2 \text{ g kg}^{-1} \text{ d}^{-1}$ ). The mononuclear cells were isolated. CD3, CD4, CD8, and cell activation marker CD69 in mononuclear cells were visualized by fluorescent-labeled antibodies (Biolgend, California, United States) and subjected to LSRII flow cytometer. Data were analyzed using FlowJo software v10.

## Transcriptomics Analysis for Gene Expression Comparison

Total cell RNA were isolated from LWWL-treated, TDF-treated, and untreated HepG2.2.15 cells (named as LWWL group, TDF group and control group, respectively) and subjected to Agilent GeneChip (Shanghai Oebiotech Company, Shanghai, China) for transcriptomics analysis as previously described (Cheng et al., 2014). In brief, the differentially-expressed genes (DEGs) were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) database and gene ontology (GO) in the DAVID 6.8 (<https://david.ncifcrf.gov/tools.jsp>) to identify genes involved in HBV infection-related molecular interaction network. Transcriptomics data has been successfully deposited and is public. The accession number is GSE183509.



## RNA Quantification by qRT-PCR

The relative expression level of RNA of 24 DEGs was quantified using qRT-PCR, with  $2^{-\Delta\Delta CT}$  method using  $\beta$ -actin as a reference control (Livak and Schmittgen, 2001). The primers are used in qPCR shown in **Supplementary Table S1**. TransStart Green RT-qPCR kit (Transgen Biotech Co., LTD., Beijing, China) was used to determine RNA level.

## Network Pharmacology Analysis for Active Compounds of LWWL and Targets of HBV

Firstly, the six herb names in LWWL were put into the traditional Chinese medicine systems pharmacology database (TCMSP) respectively to extract compounds of all herbs, the website address of TCMSP is <http://tcmspw.com/>. Furtherly, possible active compounds were filtered based on their corresponding pharmacodynamic parameters including drug-like (DL) and oral bioavailability (OB), and “DL  $\geq 0.18$ , OB  $\geq 30\%$ ” was used as screening conditions. Secondly, possible active compounds screened by OB and DL in LWWL were paired with potential target proteins based on TCMSP database, and the Uniprot database (<https://www.uniprot.org/>) was used to obtain the gene names of target proteins. Finally, Genecards database (<https://www.genecards.org/>) was used to extract HBV-related targets according to key words species “homo species” and “hepatitis B”.

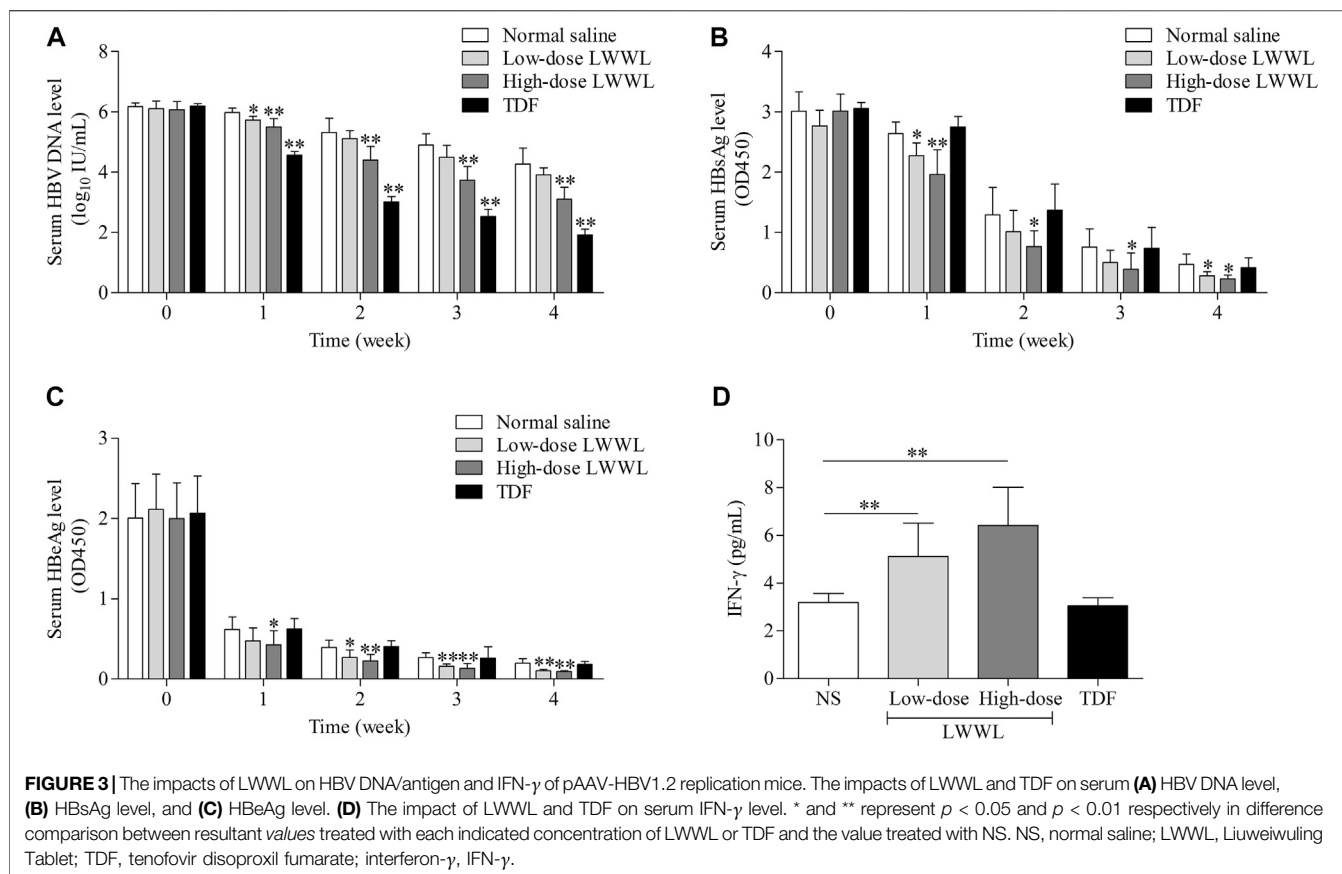
## LWWL-Compounds-Target-HBV Network Analysis

The “LWWL-compounds-targets-HBV” interaction network was constructed using Cytoscape software (Version 3.7.2) on the basis of the intersection of LWWL-related potential targets and HBV-related molecules. The value of degree was calculated to evaluate the anti-HBV potential of different compounds in LWWL. Targets of LWWL against HBV-related molecules through Cytoscape software analysis were placed in the DAVID 6.8 database for KEGG and GO analyses.

## Liquid Chromatograph-Mass Spectrometer Analysis of LWWL

The LWWL was qualitatively and quantitatively determined via LC-MS. The chromatographic conditions as below: Column, Phenomenex Kinetex 2.6u Bi-phenyl 100A, 50 × 3 mm; mobile phase A (water with 0.1% FA) and B (acetonitrile with 0.1% FA); elution program (0–0.5 min, 10% B; 0.5–4.0 min, 40% B; 4.0–9.0 min, 90% B; 12.0 min, 90% B; 12.0–15.0 min, 10% B). Flow rate was 0.4 ml/min; and injection volume was 5.0  $\mu$ l. Electrospray positive ionization mode was used for analysis. The mass spectrometer was operated in positive mode with the main parameters set as follows: GS1 was 50 psi; GS2:50 psi; Curtain gas (N<sub>2</sub>) pressure was 35 psi; collision gas was nine psi; and capillary temperature was 550°C.





## Statistical Analysis

SPSS16.0 software was used for statistical analysis. The data are expressed as the mean  $\pm$  standard deviation, and the experimental groups and the control group were analyzed by a *t*-test. Other data were analyzed by one-way analysis of variance (ANOVA). A *p* value  $< 0.05$  was considered statistically significant.

## RESULTS

### Antiviral Effect of LWWL in Cell Models

Cell viability kept well when LWWL concentration was  $\leq 0.8$  mg/ml of LWWL. The  $CC_{50}$  values were 3.14 mg/ml and 4.57 mg/ml in both cell models (Figures 1A,C), respectively. Under the safe concentration, the antiviral effect of LWWL was evaluated on the first, third and fifth days respectively, and it was found that antiviral effect of LWWL was the best on day 5 of the treatment in both cell models (Figures 1B,D).

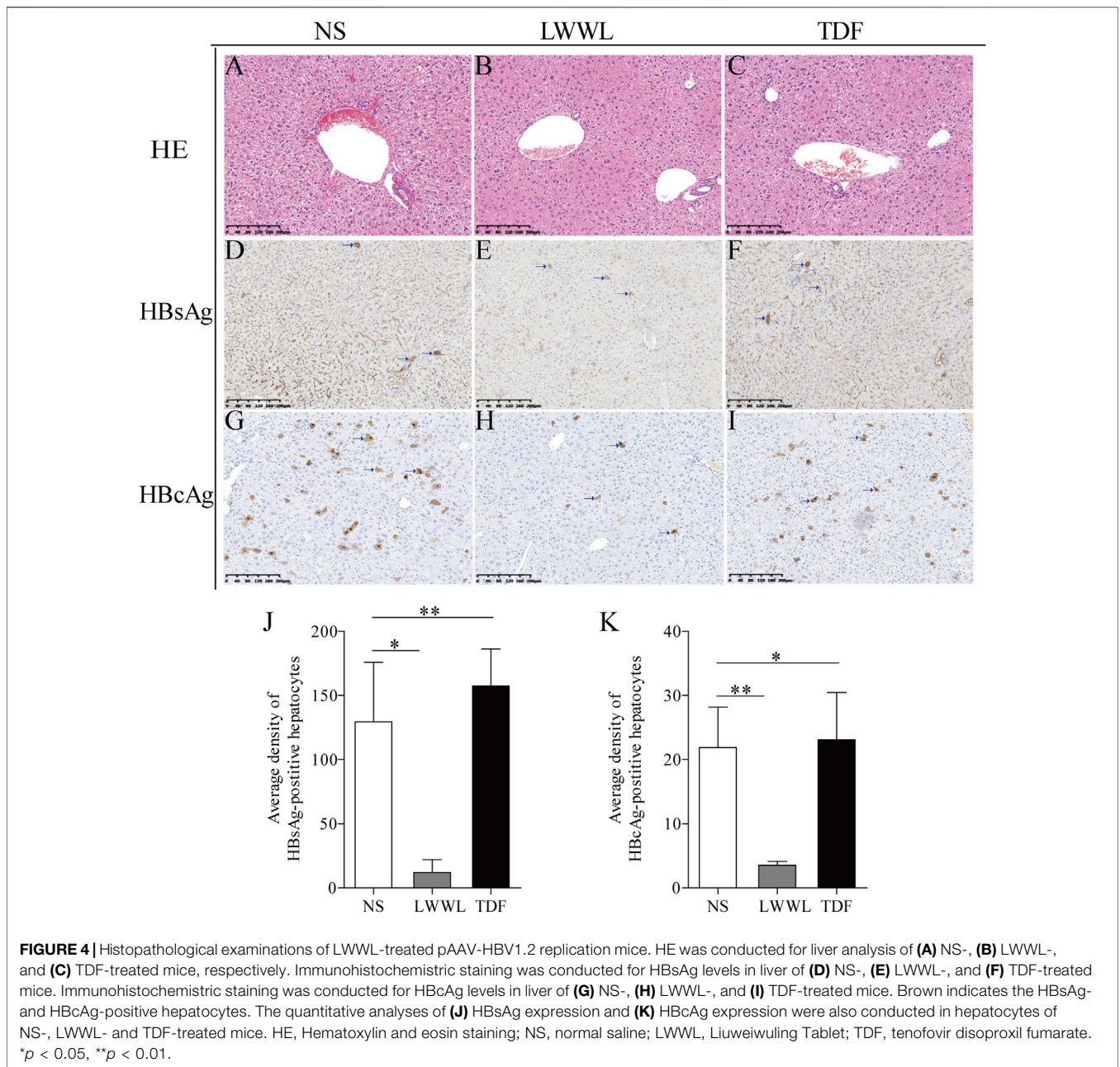
In wild-type HBV-replicating HepG2.2.15 cells, the inhibitory rates of LWWL (0.8 mg/ml) on HBV DNA, HBsAg, HBeAg and pgRNA were 57.06, 38.55, 21.26, and 62.49%, respectively (Figures 2A,C). By contrast, the inhibitory rates of TDF (200  $\mu$ mol/L) on HBV DNA, HBsAg, HBeAg, and pgRNA

were 86.18, 13.91, 12.66, and 45.55%, respectively (Figures 2B,C). In ETV-resistant HBV-replicating HepG2.A64 cells, the inhibitory rates of LWWL (0.8 mg/ml) on HBV DNA, HBsAg, and pgRNA were 51.57, 17.57, and 53.88%, respectively (Figures 2D,F). By contrast, the inhibitory rates of TDF (200  $\mu$ mol/L) on HBV DNA, HBsAg, and pgRNA were 80.20, 13.26, and 31.93%, respectively (Figures 2E,F).

### Antiviral Effect of LWWL in Mouse Model

In 4-weeks treatment, serum HBV DNA levels were decreased 0.36, 1.16, and 2.35  $\log_{10}$  IU/ml in low-dose LWWL-, high-dose LWWL-, and TDF-treated mice compared to NS-treated mice. Serum HBeAg levels were decreased 47.47, 53.20, and 8.26%, and serum HBsAg levels were decreased 40.28, 50.77, and 11.53%, respectively post 4-weeks treatment (Figures 3A–C). Compared to NS-treated mice, serum IFN- $\gamma$  levels of low-dose LWWL, high-dose LWWL, and TDF-treated mice had 1.60-, 2.01-, and 0.95-fold increases, respectively (Figure 3D).

The average densities of HBsAg-positive hepatocytes in the NS-, high-dose LWWL-, and TDF-treated mice were  $129.48 \pm 46.24$ ,  $12.02 \pm 9.89$ , and  $157.31 \pm 29.05$ , respectively (Figures 4D–F). Average densities of HBeAg-positive hepatocytes of NS-treated, high-dose LWWL-treated, and TDF-treated mice were  $21.88 \pm 6.31$ ,  $3.54 \pm 0.59$ , and  $23.07 \pm 7.40$ , respectively (Figures



**FIGURE 4 |** Histopathological examinations of LWWL-treated pAAV-HBV1.2 replication mice. HE was conducted for liver analysis of (A) NS-, (B) LWWL-, and (C) TDF-treated mice, respectively. Immunohistochemistic staining was conducted for HBsAg levels in liver of (D) NS-, (E) LWWL-, and (F) TDF-treated mice. Immunohistochemistic staining was conducted for HBcAg levels in liver of (G) NS-, (H) LWWL-, and (I) TDF-treated mice. Brown indicates the HBsAg- and HBcAg-positive hepatocytes. The quantitative analyses of (J) HBsAg expression and (K) HBcAg expression were also conducted in hepatocytes of NS-, LWWL- and TDF-treated mice. HE, Hematoxylin and eosin staining; NS, normal saline; LWWL, Liuweiwuling Tablet; TDF, tenofovir disoproxil fumarate. \**p* < 0.05, \*\**p* < 0.01.

4G–I). Statistical analysis showed that the number of the HBV antigen-positive hepatocytes was significantly decreased in LWWL-treated mice than in TDF-treated and NS-treated mice (Figures 4J,K).

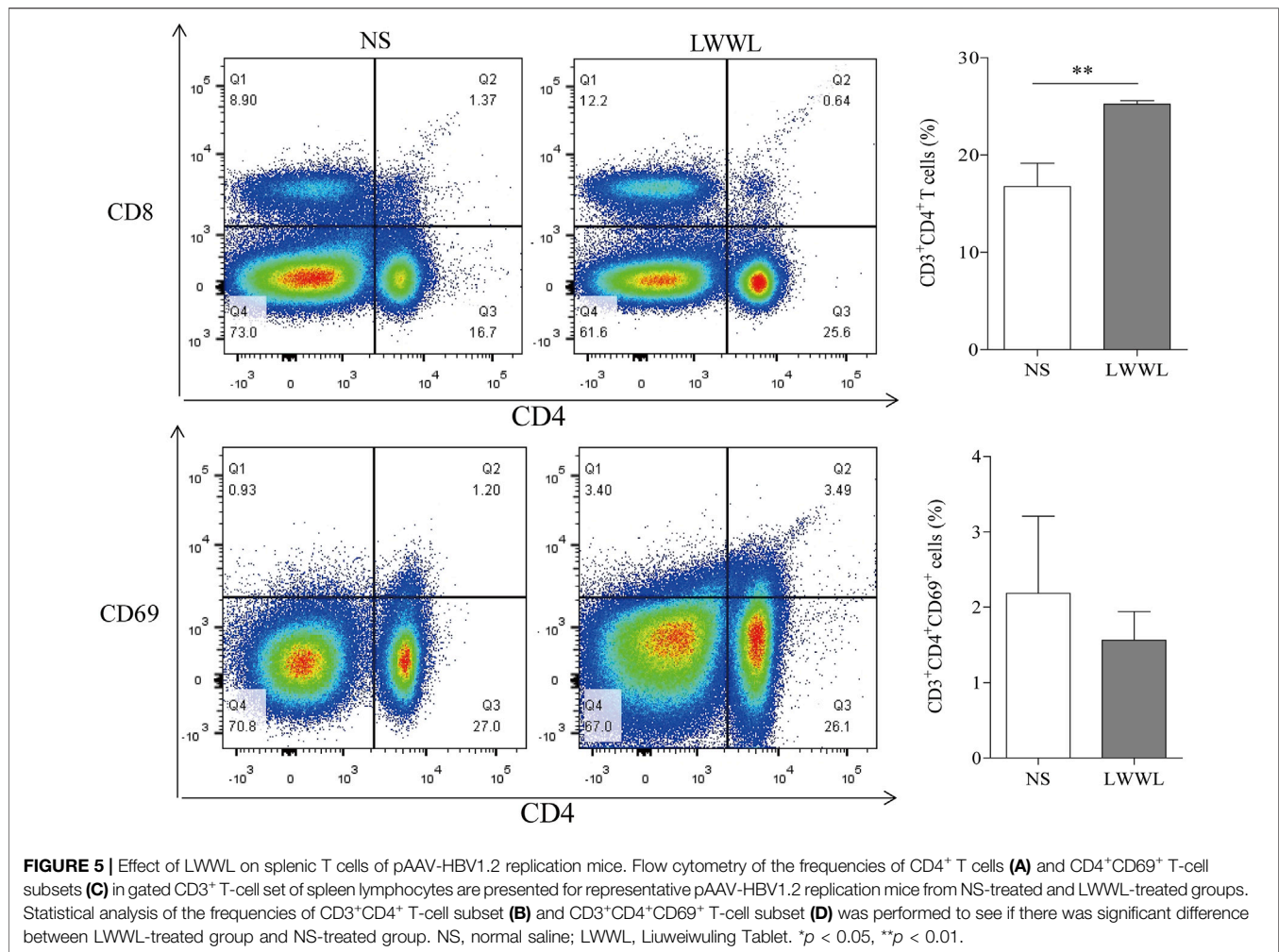
**Effect of LWWL on Splenic T Cells Activation**

The frequency of CD3<sup>+</sup>CD4<sup>+</sup> T cells in high-dose LWWL-treated mice was significantly higher than that in NS-treated mice (17.10 ± 1.95% vs 25.43 ± 1.28%, *p* < 0.01) (Figures 5A,B); whereas no significant difference was observed for the frequencies of CD3<sup>+</sup>CD4<sup>+</sup>CD69<sup>+</sup> T cells between LWWL-treated and NS-

treated mice (1.97 ± 0.82 vs 1.63 ± 0.54%, *p* > 0.05) (Figures 5C,D).

**Differential Gene Expression in Transcription Level Based on Transcriptomics and qRT-PCR Verification**

There were 2,074 up-regulated genes and 985 down-regulated genes in LWWL-treated HepG2.2.15 cells compared with untreated cells. Total of 24 DEGs was found to be involved in HBV-related pathway by KEGG pathway analysis in LWWL-treated cells compared to control group (Figures 6A–D).



Among them, 13 DEGs between LWWL group and control group were verified by RT-qPCR. Compared to control group, EGR2 and FOS expression in LWWL group were significantly lower, IKBKE, CCNE2, AKT3, CREB3L2, PIK3R3, CREBBP, BCL2, PCNA, E2F1, CASP8 and P53 expression in LWWL group were significantly higher (Figure 6E). In addition, IFN- $\beta$  expression levels in LWWL group were significantly higher than that in control group (Figure 6F).

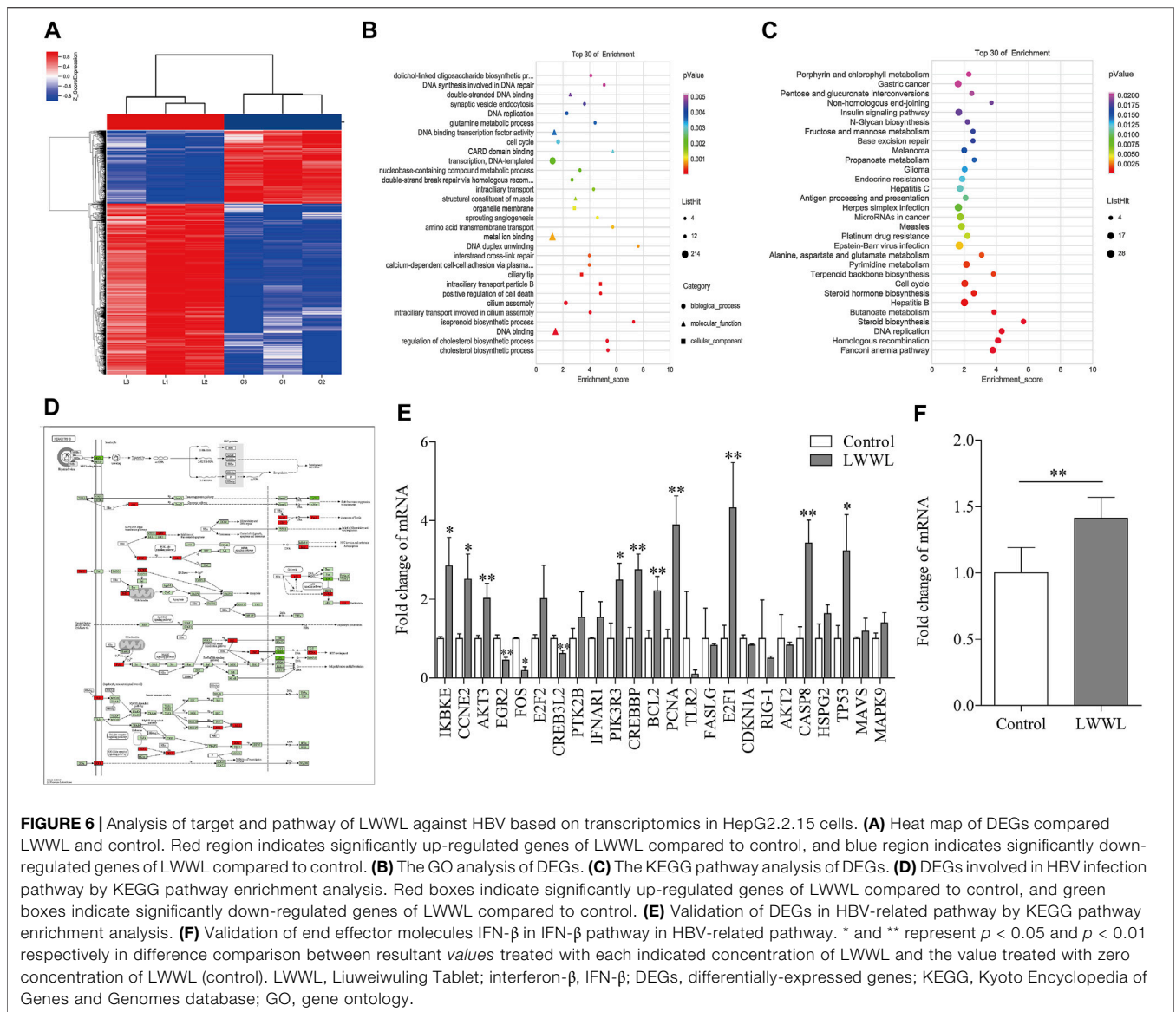
### Experimental Verification of Major Active Compounds of LWWL Associated with HBV Inhibition Based on Network Pharmacology Prediction

Total of 35 compounds contained in LWWL were subjected to network pharmacology prediction that contained 2107 HBV-related targets. As a result, 26 active compounds were found to be involved 128 HBV-related targets (Figure 7; Table 1).

Among the 26 potential anti-HBV compounds, seven compounds (quercetin, kaempferol, luteolin, wogonin,

beta-sitosterol, bicuculline, and lucidumoside D-qt) had >10 degree value were taken into further experimental analysis as a higher degree usually indicates a greater potential for anti-HBV activity. Among the seven compounds, quercetin, luteolin, wogonin, and kaempferol showed better anti-HBV effects. The molecular structures of the four active compounds are shown in Figure 8A1, B1, C1, D1. Cytotoxic testing showed the four compounds are shown in Figure 8A2, B2, C2, D2. The maximum concentrations with anti-HBV effects *in vitro* were 5.00  $\mu\text{mol/L}$  for quercetin, 2.50  $\mu\text{mol/L}$  for luteolin, 2.50  $\mu\text{mol/L}$  for wogonin, and 6.25  $\mu\text{mol/L}$  for kaempferol, respectively. As a result, the inhibitory rates on supernatant HBV DNA in HepG2.2.15 cells were 53.47, 53.28, 54.05, and 28.93% for quercetin, luteolin, wogonin, and kaempferol, respectively; and the inhibitory rates on HBsAg/HBeAg for the four compounds were 38.04%/14.25%, 45.00%/36.38%, 19.41%/15.95 and 19.35%/23.02%, respectively (Figure 8A3, B3, C3, D3). Furthermore, we have identified those four active compounds in LWWL based on LC-MS analysis (Table 2).





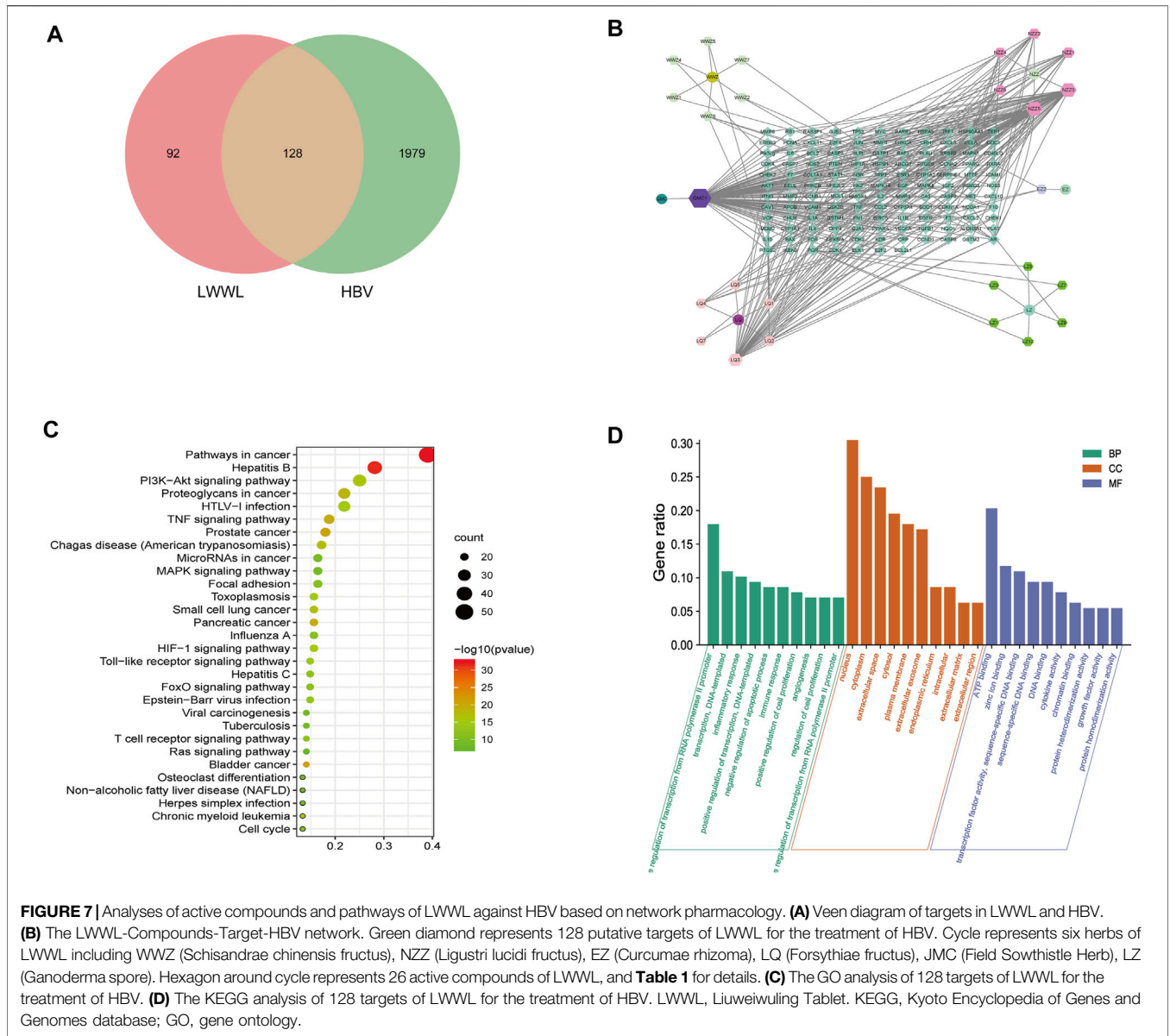
## DISCUSSION

The major drawback for current anti-HBV agents is that they do not effectively eliminate HBV from patients with chronic HBV infection. NAs are the most commonly-used antiviral agents. They effectively inhibit viral replication but has no direct suppressive effect on covalently closed circular DNA (cccDNA), and the suppressive effect is much weaker on HBV antigen expression than on HBV replication (Chevaliez et al., 2013). In addition, long-term use of NAs may induce HBV drug-resistance (Terrault et al., 2018). The life of HBV cycle and viral interplay with host involve multiple factors. Thus, agents reactive to multiple targets are required for effectively eliminating the virus (Wang, et al., 2021; Terrault et al., 2018). Because Chinese patent medicine contains multiple active components against multiple targets related to HBV proliferation, they may have a potential superior in playing multi-

target synergistic antiviral effects (Liu et al., 2018; Hepatobiliary Specialized Committee of China Association of Chinese Medicine and Liver Diseases Specialized Committee of China Medical Association of Minorities., 2018). Clinical use of LWWL has shown that it is well efficacious on anti-inflammation of chronic HBV infection (Hepatobiliary Specialized Committee of China Association of Chinese Medicine et al., 2020). In addition, a meta-analysis documented that combination of LWWL with NAs (at least for 3 months) could increase the rate of HBV DNA undetectability ( $OR = 1.8\text{--}6.71$ ,  $p < 0.05$ ) and HBeAg loss ( $OR = 1.83\text{--}2.04$ ,  $p < 0.05$ ) compared to single use of NAs (He et al., 2017; Wang et al., 2020). So far, there is still lack of data on experimental anti-HBV effects and underlying mechanisms of LWWL.

Our study showed LWWL had anti-HBV effect both for wild-type and for ETV-resistant viruses. Compared to TDF





control, LWWL was more efficient in suppressing HBV antigen levels, although it was less efficient in suppressing HBV DNA level. This antiviral effect was verified in HBV-replicating mouse. HBV antigen such as HBsAg is critical for HBV to establish immune tolerance, which could facilitate the persistence of HBV infection by suppressing host immunity through the regulation of IFN-related pathway (Jiang et al., 2014; Warner et al., 2020). LWWL had a better efficacy in suppression HBsAg production and this endow it potential to play a synergistic effect with NAs. In addition, compared to TDF, LWWL had a better effect on inhibiting HBV pgRNA of both wild-type and ETV-resistant HBV. As pgRNA is directly transcribed from cccDNA, it may more closely reflect cccDNA activity compared to the other viral markers (Wang et al., 2017).

Transcriptomics provides a novel and effective way to refine clues about complex mechanisms. Therefore, we used transcriptomics to analyze the potential mechanisms of LWWL against HBV. Transcriptomics analysis and qRT-PCR verification showed that there were 13 DEGs at transcription levels involved in HBV-related molecular pathways, mainly including P53, apoptosis, and IFN-β pathways. Further analysis showed that the expression level of effector IFN-β in IFN-β pathway were significantly higher in LWWL-treated HepG2.2.15 cells than that in LWWL-untreated cells. IFN-β has been verified in previous studies through activating pathogen-associated molecular patterns (such as TLRs, RIG-1, c-GAS)/IFN-β pathways (Yin et al., 2016; Cheng et al., 2017; Alexopoulou et al., 2020). Our results also showed that in HBV-replicating mouse model, LWWL

**TABLE 1** | Prediction of anti-HBV components of LWWL based on network pharmacology.

Composition	Degree	OB%	DL%
quercetin	97	46.43	0.28
kaempferol	46	41.88	0.24
luteolin	45	36.16	0.25
wogonin	33	30.68	0.23
beta-sitosterol	13	36.91	0.75
bicuculline	12	69.67	0.88
Lucidumoside D_qt	12	54.41	0.47
taxifolin	9	57.84	0.27
Onjixanthone I	8	79.16	0.3
eriodictyol	7	71.79	0.24
(+)-pinoresinol monomethyl ether	6	53.08	0.57
(3R,4R)-3,4-bis[(3,4-dimethoxyphenyl)methyl]oxolan-2-one	6	52.3	0.48
Gomisin R	5	34.84	0.86
hederagenin	4	36.91	0.75
Angeloylgomisin O	3	31.97	0.85
Mairin	2	55.38	0.78
campesta-7,22E-dien-3beta-ol	2	43.51	0.72
ergosta-7,22E-dien-3beta-ol	2	43.51	0.72
ergosta-4,6,8(14),22-tetraene-3-one	2	48.32	0.75
ganoderol B	2	42.56	0.81
Lucialdehyde B	2	43.12	0.81
lucidone A	2	37.22	0.64
Gomisin-A	2	30.69	0.78
Wuweizisu C	2	46.27	0.84
Schizandrer B	2	30.71	0.83
Deoxyharringtonine	2	39.27	0.81

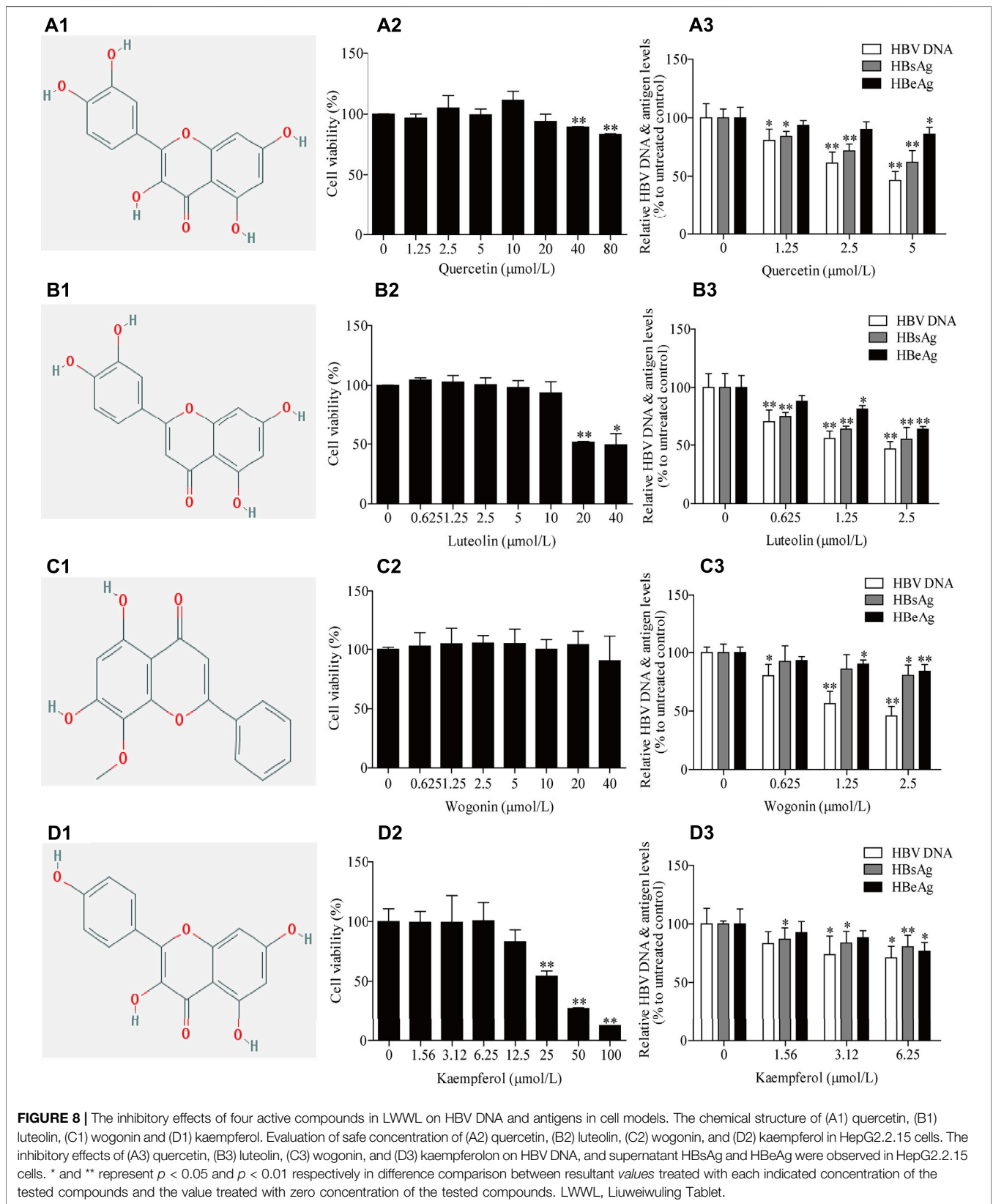
treatment significantly increased the frequency of CD3<sup>+</sup>CD4<sup>+</sup> T cells and serum IFN- $\gamma$  production. IFN- $\gamma$  is mainly generated by activated CD3<sup>+</sup>CD4<sup>+</sup> T cells, and IFN- $\gamma$  has been proved to be able to inhibit HBV replication through triggering intracellular antiviral pathways (Chokshi et al., 2014; Sang et al., 2017; Iannacone and Guidotti, 2021). We speculated that LWWL might play an anti-HBV role by enhancements of both IFN- $\beta$ -mediated innate anti-HBV effect and IFN- $\gamma$ -mediated acquired anti-HBV effect, whereas further studies are needed for the confirmation and elucidation of the context of the pathways.

Network pharmacology, developed in recent years, is an integration of bioinformatics and pharmacology by constructing the network of Chinese medicine-compounds-target-disease (Yang et al., 2018; Zhang et al., 2019). It has been proved to be a useful tool for predicting active compounds of Chinese medicine against certain diseases. Therefore, we firstly predicted the potential active compounds against HBV in LWWL based on network pharmacology, and then focused on the compounds with high degree that usually indicates a greater potential for anti-HBV activity. Out of the 26 compounds with potential anti-HBV effects that were predicted by network pharmacology, four compounds (quercetin, luteolin, wogonin, and kaempferol) were experimentally confirmed to have antiviral potency, which partly clarified the active compounds of LWWL against HBV, and also provides a reference for the new drug screening of HBV. Previous studies showed that luteolin

had inhibitory effect on HBV replication through regulating HNF 4 $\alpha$  expression (Bai et al., 2016), and quercetin could inhibit HBV DNA, HBsAg, and HBeAg levels *in vitro* but mechanisms remained clarification (Cheng et al., 2015). Our team reported that wogonin was one of major active compounds against HBV in Chinese herbal extracts Suxing, and wogonin had inhibitory effects on HBV cccDNA in addition to regular HBV DNA and antigens (Liu et al., 2018; Si et al., 2019). In this study, we confirmed that quercetin, luteolin, and wogonin in LWWL had anti-HBV effects, and found that kaempferol in LWWL was also an anti-HBV component. These enriched the knowledge on the anti-HBV effects of LWWL and provided a good start for elucidating the antiviral mechanisms of LWWL, although it is still a way to go to comprehensively elucidate the antiviral mechanisms of LWWL.

## CONCLUSION

In this study, we for the first time found that Chinese patent medicine LWWL could effectively suppress the activities of both wild-type and ETV-resistant HBV in cell models and the suppressive effects were superior to TDF on HBsAg expression. The antiviral effects were also verified in HBV-replicating mouse model. Our study suggested that LWWL against HBV might be associated with increasing IFN- $\beta$  and IFN- $\gamma$  productions. Four major active anti-HBV compounds



**TABLE 2** | Identification of the compounds of LWWL in chromatography.

Index	Component name	Formula	Precursor Mass	Found at Mass	Mass error (ppm)
1	luteolin	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	287.055	287.0553	0.9
2	quercetin	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	303.05	303.0504	1.5
3	wogonin	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	285.076	285.076	1
4	kaempferol	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	287.055	287.0554	1.5

from LWWL were firstly identified. These findings can provided new insights into the anti-HBV activities of LWWL, which may help optimize combination therapy of LWWL with current NAs and develop novel LWWL-derived anti-HBV agents.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/geo/>, GSE183509.

## ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Ethics of Animal Experiments of The Fifth Medical Center of Chinese PLA General Hospital (Permit number: IACUC-2021-0009).

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## AUTHOR CONTRIBUTIONS

X-HX, YL, and Z-FB were responsible for the design of the whole experiment. F-LG, L-LS, YY and Y-HL were responsible for data analysis and drafted the manuscript. Z-LL, W-HL and X-CL participated in Network pharmacology. Z-LZ, HL, JW and LL participated in experiment related to animal model. HL, JZ, D-PX and J-BW contributed for revising manuscript. The final version was read and approved by all authors.

## FUNDING

This work was supported by the National Natural Science Foundation of China (81930110, 81721002).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2021.756975/full#supplementary-material>



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